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Benzotriazoles Reactivate Latent HIV-1 through Inactivation of STAT5 SUMOylation

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SUMMARY

The presence of latent HIV-1 in infected individuals represents a major barrier preventingviral eradication. For that reason, reactivation of latent viruses in the presence of antiretroviral regimens has been proposed as a therapeutic strategy to achieve remission. We screened for small molecules and identified several benzotriazole derivatives with the ability to reactivate latent HIV-1. In the presence of IL-2, benzotriazoles reactivated and reduced the latent reservoir in primary cells, and,

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Conceptualization, A.B., K.A.N., D.H.P., and V.P.; Methodology, A.B. and K.A.N.; Software, K.A.N., Validation, A.B. and K.A.N., Formal Analysis, A.B. and K.A.N.; Investigation, A.B., K.A.N., A.B.M., N.M.A., R.M.V.W., L.J.M., and C.L.N.; Resources, A.M.S., M.A.S., D.M.M., and C.M.I.; Data Curation, A.B. and K.A.N.; Visualization, A.B. and K.A.N.; Writing – Original Draft, A.B.; Writing – Review & Editing, A.B., K.A.N., D.H.P., and V.P.; Funding Acquisition, A.B., D.M.M., D.H.P., and V.P. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTAL INFORMATION

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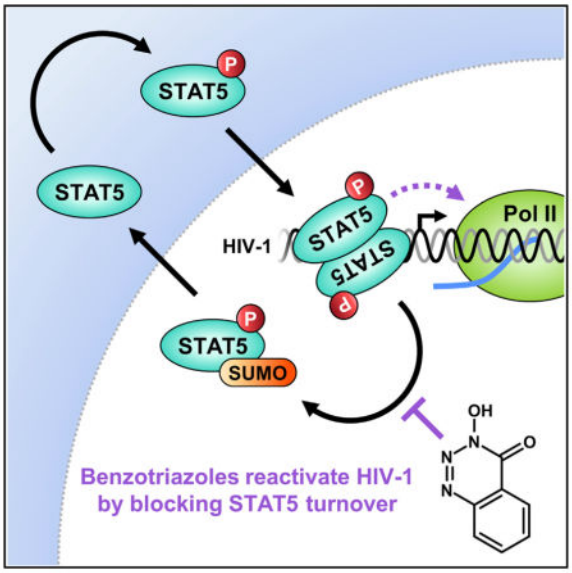
remarkably, viral reactivation was achieved without inducing cell proliferation, T cell activation, or cytokine release. Mechanistic studies showed that benzotriazoles block SUMOylation of phosphorylated STAT5, increasing STAT5's activity and occupancy of the HIV-1 LTR. Our results identify benzotriazoles as latency reversing agents and STAT5 signaling and SUMOylation as targets for HIV-1 eradication strategies. These compounds represent a different direction in the search for "shock and kill" therapies.

Graphical abstract

Latent HIV-1 represents a barrier toward HIV-1 eradication. Bosque et al. identify a family of compounds that have the ability to reactivate and decrease latent HIV-1. These compounds block SUMOylation of STAT5 and represent a target for HIV-1 eradication strategies.



INTRODUCTION

In an effort to develop strategies that will lead to an HIV-1 cure, small molecules are being sought that can induce the expression of latent HIV-1 without fully activating T cells. Current curative strategies seek to reverse HIV-1 latency and allow specific immunotherapies to clear residual HIV-1 infection in infected patients on antiretroviral therapy (ART). In this setting, continued ART must be sufficient to prevent viral spread (Barton et al., 2013; Richman et al., 2009; Shen and Siliciano, 2008; Trono et al., 2010). Drug discovery efforts have, thus far, produced two types of latency reversing agents (LRAs) having reached human testing. First, histone deacetylase inhibitors (HDACi) such as vorinostat (SAHA), romidepsin, and panobinostat (reviewed in Spivak and Planelles, 2016; Van Lint et al., 2013) transcriptionally reactivate latent HIV-1 but do so in a non-specific manner. A second candidate, disulfiram (Antabuse), was effective as a LRA in a primary cell model of latency but failed to reduce the reservoir size when administered daily to aviremic patients over a 2-week period (Spivak et al., 2014; Xing et al., 2011). Although not in human trials at this time, protein kinase C (PKC) agonists display strong activity across cell models

of latency and in patient cells (Spina et al., 2013). The rate-limiting step toward the successful development of PKC agonists will be to produce analogs that can induce viral reactivation in the absence of cellular activation and pro-inflammatory cytokine secretion (Spivak et al., 2016). Ultimately, the discovery of additional compounds and novel cellular targets will be required toward an effective “shock and kill” regimen.

Here, we identified a family of compounds, benzotriazoles, capable of reactivating latent HIV-1 both in vitro and ex vivo. Benzotriazoles had not previously been associated with a biological function. Moreover, these compounds exert anti-latency properties in the presence of interleukin-2 (IL-2) in primary $CD4^+$ T cells. Utilizing an in vitro system, we demonstrated that HIV-1 reactivation mediated by benzotriazoles was sufficient to reduce the size of the inducible latent reservoir. Interestingly, viral reactivation induced by these compounds was not accompanied by cell proliferation, cytokine release or T cell activation. RNA sequencing (RNA-seq) identified activation of signal transducers and activators of transcription (STATs) as the main transcription factor influenced by benzotriazoles and follow-up experiments confirmed that HIV-1 reactivation is dependent on STAT5 phosphorylation. We found that benzotriazoles inhibit a negative feedback loop mediated by addition of SUMO2/3, thus sustaining STAT5 phosphorylation and activity. Finally, we discovered that manipulation of STAT5 phosphorylation using benzotriazoles can reduce the seeding of the latent reservoir. Overall, this research identifies benzotriazoles as potential LRAs and STAT5 SUMOylation as a target for HIV-1 eradication strategies.

RESULTS

Benzotriazoles Induce HIV In Vitro and Ex Vivo

We screened for small, drug-like molecules that could reactivate latent HIV-1 in a primary cell model based on the generation of latently infected, cultured central memory T cells (T_{CM}) (Bosque and Planelles, 2009, 2011). Among a library of natural products isolated by the Ireland group at the University of Utah, we found a unique compound that was able to reactivate latent HIV-1 (Figures S1A and S1B). Further analysis revealed that this activity was from 1-hydroxybenzotriazol (HOBt) (Figure S1C), and we confirmed that pure HOBt was able to reactivate latent HIV-1 in a dose-dependent manner (Figure S1D). HOBt is commonly used as an additive in peptide synthesis to increase yield and reduce epimerization (Valeur and Bradley, 2009) and, prior to this report, has not been associated with any biological function.

We then tested the ability of HOBt and several other benzotriazole derivatives to reactivate latent HIV-1 in an similar primary cell model of latency that uses a replication competent virus and ART to control viral spread (Martins et al., 2016). We compared HOBt to benzotriazole (HBt), 1-hydroxy-7-amino benzotriazole (HOAt), and 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HODHBt) (Figures 1A and S2A). With the exception of HBt, all tested analogs reactivated latent HIV-1 in a dose-dependent manner. This model requires the continuous presence of IL-2 in the culture to promote the survival of CD4 T cells. We then analyzed whether viral reactivation was dependent on IL-2 in the primary cell model of latency. We tested HODHBt either alone or in the presence of IL-2. Interestingly, HODHBt only had activity in the presence of IL-2 (Figure S2B). Analysis of the relative potency of

these compounds revealed that HODHBt and HOAt were the most active. HODHBt activity was about two orders of magnitude more potent than our original hit, HOBt (Figure S2C).

The efficacy of benzotriazoles to reactivate latent HIV-1 was then evaluated using cells isolated from aviremic HIV-infected donors. Cells from four patients were examined using a modification of the REVEAL assay (Spivak et al., 2015) in which viral release was measured using a digital ELISA for HIV-1 p24 that allows detection down to 2.5 fg/mL p24 (equivalent to 63 HIV-1 RNA copies/mL) (Cabrera et al., 2015; Chang et al., 2013). The combination of IL-2 plus 100 μM HODHBt increased viral release on average 2.7-fold compared to unstimulated cells (Figure 1B). We further characterized the ability of benzotriazole derivatives to reactivate latent HIV-1 from aviremic patients using the quantitative viral outgrowth assay (QVOA) (Archin et al., 2008). HOAt induced viral release above the DMSO control in cells from both patients tested (Figure S2D). Next, we assessed the toxicity of benzotriazoles in resting CD4 T cells from four aviremic patients, by measuring caspase-3 activation, a biomarker of apoptosis, using flow cytometry. HODHBt did not induce caspase-3 activation over background (unstimulated cells) either alone or in the presence of IL-2 using concentrations up to 100 μM (Figure 1C). Finally, HODHBt failed to reactivate latent HIV-1 in four J-Lat clonal cell lines tested (Figure S2E). However, this is not surprising as benzotriazoles have anti-latency activity only in the presence of IL-2. J-Lat cells are derived from Jurkat T cells (Jordan et al., 2003), which do not express CD25, the high-affinity receptor for IL-2 and thus are insensitive to this cytokine (Ito et al., 2011).

HODHBt Reduces the Size of the Latent HIV Reservoir In Vitro

Latently infected cells in vivo appear to react in two different modes when exposed to LRAs ex vivo (Ho et al., 2013). While a minority of cells is sensitive to reactivation conditions and produces virus that can be detected by QVOA, a majority of cells appears highly resistant to the same stimulation (Ho et al., 2013). To determine whether HODHBt treatment reduced the size of the inducible reservoir, we adapted our T_{CM} model of latency (Martins et al., 2016) and asked whether a 2-day treatment with HODHBt or αCD3/αCD28 had a significant effect on the size of the αCD3/αCD28-inducible reservoir (Figure 2A). Representative results from one of five donors are depicted (Figure S3). After generation of latently infected cells (day 17), cells were treated with IL-2 alone (baseline condition) or IL-2 plus 100 μM of HODHBt or αCD3/αCD28 for 48 hr. To avoid viral spread, we cultured the cells in the presence of ART at all times (1 μM Raltegravir and 0.5 μM Nelfinavir). As expected, HODHBt caused a significant increase in p24 positive cells over IL-2-treated cells, albeit at a lower level than cells induced with αCD3/αCD28 (Figure 2B). Following stimulation, treated cells were cultured for an additional five days in the absence of any stimulus, and then stimulated with αCD3/αCD28 such that any remaining inducible reservoir would be reactivated (Figure 2A, day 24). During this second stimulation with αCD3/αCD28, cells previously stimulated with only IL-2, the baseline condition, exhibited the largest amount of reactivation (Figure 2C). Importantly, pre-stimulation of cells with IL-2 plus HODHBt led to a 44% ± 8.5% reduction relative to IL-2 treatment alone of the inducible reservoir (Figure 2C). Finally, αCD3/αCD28 treatment led to a 97.8% ± 6.5% reduction relative to IL-2 treatment alone of the inducible reservoir in this in vitro model and no significant reactivation was observed after a second αCD3/αCD28 stimulation (Figure

2C). These results demonstrate that HODHBt-induced HIV-1 reactivation is capable of reducing the latent reservoir in vitro.

Effects of HODHBt on Cellular Activation

Having confirmed the ability of HODHBt to reactivate latent HIV-1, we next examined the impact of HODHBt on cellular gene expression. T_{CM} from three independent donors were treated with IL-2 alone, IL-2 plus HODHBt, or αCD3/αCD28, and then polyadenylated RNAs were isolated and sequenced. When comparing the average gene expression changes between treatments and controls, HODHBt significantly altered fewer genes than αCD3/αCD28 (394 versus 8,257, $q \leq 0.01$), and these changes were much lower in magnitude (Figure S4). This difference in magnitude was easily appreciated when genes significantly altered by one or both treatments were plotted on a $\log_2$ graph (Figure 3A). Although 82% of the genes whose expression was changed by HODHBt were also significantly influenced by αCD3/αCD28 (Figure 3A, purple dots), approximately half of the genes upregulated by HODHBt were not upregulated by αCD3/αCD28 (101 of 223 genes). Importantly, several key markers of T cell activation (such as upregulation of IL-2 and MIR155HG, or downregulation of KLF2) were not significantly altered by HODHBt (Figure 3A; Table S1). This result indicates that the transcriptional changes induced by HODHBt are drastically different from those induced by TCR engagement.

We further evaluated whether HODHBt had the ability to activate T cells via the cell-surface markers, CD69 and CD25. While αCD3/αCD28 treatment vigorously induced the expression of CD69 and CD25 as observed via both mRNA and protein levels (Figures 3B and 3C), HODHBt treatment had minimal effects on both markers. Homeostatic proliferation of resting CD4 T cells has been proposed as a mechanism for the expansion of the latent reservoir (Bosque et al., 2011; Chomont et al., 2009; Simonetti et al., 2016). Therefore, we tested the abilities of different benzotriazoles to induce cell proliferation of cultured T_{CM} via carboxyfluorescein succimidyl ester (CFSE) staining. CFSE staining intensity dilutes to half after each cell division to the daughter cells (Renno et al., 1999). After 48 hr of treatment, none of the compounds tested in conjunction with IL-2, nor IL-2 alone (baseline condition), induce any detectable cell proliferation. αCD3/αCD28 stimulation, by contrast, induce massive cell proliferation (Figure 3D). Finally, we assessed whether HODHBt could induce global immune activation by measuring the release of pro-inflammatory cytokines, a potential undesirable side effect of therapies aimed toward HIV-1 eradication (Prins et al., 1999). To that end, we measured a panel of 13 cytokines released from PBMCs from five healthy donors. Phorbol 12-myristate 13-acetate (PMA) plus ionomycin (PMA/I) was used as a positive control and induced the tested cytokines to levels above those observed in unstimulated controls (Figure 3E, “none”). With the exception of low levels of IL-6, HODHBt did not induce the release of any cytokine above baseline (Figure 3E). We also analyzed the induction of these cytokines in $CD4^+$ T cells in our RNA-seq data. In contrast with αCD3/αCD28, which induced 12 of the 13 cytokines over the IL-2 treatment baseline, none of the cytokines showed any statistically significant difference between IL-2 and IL-2 plus HODHBT treatments (Figure S5). Although interferon (IFN)-γ and IL-9 were induced by IL-2 plus HODHBt in one of the three donors, this was not statistically significant due to variation between the three donor samples (Table S1).

Overall, these results indicate that HODHBt does not alter the expression of key T cell activation regulators and surface markers, does not induce cellular proliferation, and does not promote cytokine release. Therefore, we conclude that HODHBt can induce viral reactivation in the absence of substantial T cell and immune activation.

Benzotriazoles Prolong STAT5 Activation

We sought to determine the transcription factor(s) responsible for the changes in gene expression triggered by HODHBt. The 394 genes differentially expressed in HODHBt-treated cells were analyzed using the software program, Predicting ASsociated Transcription factors from Annotated Affinities (Roider et al., 2009). PASTAA predicted the involvement of signal transducers and activators of transcription (STATs) (Table S2). This was confirmed by testing the ability of known STAT inhibitors to block HOAt-mediated viral reactivation (Figure S6A). Janus kinases (JAK) activate STATs. DBI, a pan-JAK inhibitor, significantly reduced viral reactivation mediated by HOAt. Furthermore, STAT5 but not STAT3 inhibition abrogated viral reactivation mediated by HOAt (Figure S6A). In contrast, BMS245541 or Cyclosporin A, inhibitors of nuclear factor κB (NF-κB) and NFAT, respectively, had no significant effects (Figure S6A). Based on the above results and a previous report suggesting that STAT5 modulates viral transcription in CD4 T cells (Selliah et al., 2006), we hypothesized that STAT5 plays a direct role in benzotriazole-mediated reactivation of latent HIV-1.

STAT5 activation and dimerization is associated with tyrosine phosphorylation on Y694 by JAK kinases (Gouilleux et al., 1994). To evaluate the effects of benzotriazoles on the dynamics of STAT5 phosphorylation, we measured STAT5 Y694 phosphorylation levels (pSTAT5) by flow cytometry over a 24-hr time course of cultured T_{CM} treated with HOAt, IL-2, or a combination of both (Figure S6B). IL-2 induced STAT5 phosphorylation after 30 min (34% of cells), but this phosphorylation was short lived as pSTAT5 levels rapidly fell over all subsequent time points (Figure S6B, IL-2). The combination of IL-2 and HOAt, however, resulted in elevated pSTAT5 levels throughout the entire 24-hr window (42.6%, 53.3%, 62.8%, and 52.8% of cells after 0.5, 3, 6, and 24 hr) (Figure S6B, IL-2 + HOAt). Interestingly, HOAt did not induce STAT5 phosphorylation in the absence of IL-2 (Figure S6B, HOAt). These data indicate that HOAt alone does not induce STAT5 phosphorylation but instead stabilizes pSTAT5 levels after stimulation with IL-2. We then tested the ability of other benzotriazoles to maintain pSTAT5 levels in cultured T_{CM}. After 24 hr, all compounds tested, except for HBt, which is unable to reactivate latent HIV, had the ability to sustain STAT5 phosphorylation to varying degrees (Figure 4A). Critically, the differential abilities of these compounds to maintain STAT5 phosphorylation over time directly correlated with the extent to which they reactivated latent HIV-1 (Figure 4B). Therefore, we propose that active benzotriazole derivatives reactivate latent HIV-1 by maintaining STAT5 in its active, phosphorylated form.

To determine whether HODHBt-induced reactivation of HIV-1 is a direct consequence of STAT5 binding to the HIV-1 LTR, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) against STAT5A in latently infected T_{CM} incubated with DMSO (untreated), IL-2 plus DMSO, or IL-2 plus 100 μM HODHBt for 3 hr, and the resulting

paired-end reads were mapped to the HIV-1 genome. After HODHBt treatment, a distinct peak of STAT5A appeared at position 329, and this peak was centered between two STAT5 binding sequences that lie an appropriate distance apart to enable STAT5 tetramerization (Lin et al., 2012) (Figure 4C). Notably, STAT5 has been previously reported to bind to this region both in vitro and in infected cells (Selliah et al., 2006). This result indicates that STAT5A is specifically recruited to the HIV-1 LTR promoter more efficiently after HODHBt treatment and further supports a model where STAT5 can directly reactivate HIV-1.

We then sought to address the mechanism of action (MOA) of benzotriazoles. STAT family proteins are phosphorylated by JAK family kinases. In the case of IL-2 stimulation, JAK1 and JAK3 have been associated with STAT5 activation (Nelson and Willerford, 1998). We analyzed whether HOAt affected the phosphorylation of JAK1 and JAK3 after IL-2 treatment. Both, JAK1 and JAK3 were quickly phosphorylated after a 15-min treatment with IL-2, and the phosphorylation levels were drastically diminished thereafter. The presence of HOAt did not alter the activation of either JAK kinase (Figure S6C).

Acetylation and SUMOylation have opposite effects on STAT5 activity. Acetylation is required for phosphorylation while SUMOylation leads to a reduction of STAT5 phosphorylation and further downstream signaling (Van Nguyen et al., 2012). To determine whether benzotriazoles would affect either acetylation or SUMOylation of STAT5, cultured T_{CM} were treated with IL-2 in the presence of HODHBt or DMSO for 24 hr, and total STAT5 was immunoprecipitated using an antibody that recognizes the amino terminal region of STAT5 irrespective of the phosphorylation status. We then assessed the levels of acetylation using an antibody that recognizes acetylated lysine (acetyl-K) and the levels of SUMOylation using an antibody that recognizes SUMO2 and SUMO3. SUMO2 and SUMO3 are 95% homologous in sequence and no antibodies are currently available that distinguish between them (Kerscher et al., 2006). HODHBt reduced the levels of STAT5 SUMOylation but not acetylation when compared to those under DMSO-treated cells (Figure 5A). To determine whether the levels of SUMOylation of the phosphorylated and active STAT5 were affected, T_{CM} were cultured for 24 hr with IL-2 and either DMSO or HODHBt and then probed for pSTAT5 and STAT5. Treatment with HODHBt increased the levels of pSTAT5 without affecting the total levels of STAT5 (Figure 5B). We then immunoprecipitated pSTAT5 using an antibody that recognizes phospho-Y694 and assessed the levels of SUMOylation. HODHBt drastically reduced the levels of pSTAT5 SUMOylation when compared to those in DMSO-treated cells (Figure 5B).

Based on the above results, one should expect an increase in nuclear translocation of STAT5 in the presence of benzotriazoles. Cytoplasmic and nuclear fractions were isolated from cultured T_{CM} after treatment with IL-2 alone or in combination with HOAt at different time points. pSTAT5 nuclear levels increased after 15 min of incubation with either IL-2 alone or in combination with HOAt (Figure 5C). The levels of nuclear pSTAT5 decreased after 3 hr in cells incubated with IL-2 alone but were maintained in the presence of IL-2 plus HOAt (Figure 5C). Total levels of STAT5 were also increased in the nuclear fractions of cells treated with IL-2 plus HOAt (Figure 5C). These results indicate that HODHBt acts by blocking SUMOylation of STAT5 and pSTAT5, prolonging the nuclear retention and activity of STAT5.

Benzotriazoles Reduce the Establishment of Latency

Based on our results that benzotriazoles prolong STAT5 activity, we postulated that benzotriazoles may perturb the establishment of latency in primary CD4 T cells if applied early on during infection. To test our hypothesis, we modified our primary cell model of latency as follows. Infected cells were cultured in the absence or in the presence of 100 μM HODHBt during the active viral replication phase and prior to the introduction of ART (day 10 to day 13, Figure S7). The presence of HODHBt increased the number of p24 positive cells at day 13 (Figures 6A and S7). This increase could be the consequence of two opposing scenarios. On one hand, HODHBt could be increasing viral replication. If that were the case, we would expect an increase in the size of the viral reservoir in this cell model (Martins et al., 2016). On the other hand, HODHBt could be blocking the entry into the latent state by promoting HIV-1 expression. In this case, we would expect a reduction in the number of cells that can reactivate latent HIV-1. To ascertain which of the two mechanism was involved, we generated latently infected cultured T_{CM} from cells treated with IL-2 alone or with IL-2 plus HODHBt (Figure S7, day 17 post-sorting). We then reactivated these cultures with αCD3αCD28 to assess the percentage of latently infected cells. When cells were incubated with IL-2 alone, 3.5% ± 1.1% of cells reactivated with αCD3αCD28 (Figure 6B). In contrast, cells incubated with IL-2 in the presence of HODHBt gave rise to an average of 2.5% ± 0.9% reactivation, a 25% reduction when compared with IL-2 alone (Figure 6B). This indicated that HODHBt reduced the number of cells that harbor latent viruses in this primary cell model of latency (Figures 6 and S7). Altogether, our results point toward STAT5 as a key player in controlling not only the exit but also the entry into the latent state.

DISCUSSION

We identified benzotriazoles as a promising family of LRAs and demonstrated that they function by hindering the natural turnover of activated STAT5, which is controlled by SUMOylation. Not only do benzotriazoles present desirable therapeutic characteristics, as they fail to induce cellular proliferation, global immune activation, and T cell activation, but they also highlight STAT5 signaling as a pathway that can be exploited for “shock and kill” strategies against HIV-1. We showed that benzotriazoles impair SUMOylation of STAT5, a step required for STAT5 turnover, and this leads to increased STAT5 phosphorylation, nuclear localization and activity over time and, thus, increased transcriptional activity of the HIV-1 promoter (Figure 7). It is perhaps not unexpected that STAT5 is involved in viral reactivation. STAT5 has been reported to bind the HIV-1 LTR (Crotti et al., 2007; Selliah et al., 2006), and overexpression greatly increased p24 levels in otherwise un-stimulated HIV-infected primary T cells (Selliah et al., 2006). While it is currently unclear how HODHBt and its analogs influence STAT5 SUMOylation, a plausible target is the SUMO-conjugating enzyme Ubc9, which is thought to be responsible for STAT5 SUMOylation (Van Nguyen et al., 2012).

Our results also support the idea that modulation of STAT5 can regulate the establishment of HIV-1 latency. STAT5 protein levels were decreased in PBMCs after only 8 days of HIV infection and were reduced in T cells isolated from patients at various stages of infection (Pericle et al., 1998). Furthermore, STAT5 phosphorylation after long-term IL-2 stimulation

was also suppressed in HIV-infected patient T cells (Zheng et al., 2008). Curiously, this HIV-dependent reduction of STAT5 protein and phosphorylation levels appears to be strain specific (Pericle et al., 1998; Selliah and Finkel, 2001). Nuclear translocation of phosphorylated STAT5, a step required for its transcriptional activity, was also impaired in CD4 T cells from viremic patients (Landires et al., 2011). If suppression of STAT5 is required for HIV-1 to establish or maintain latency, compounds that directly activate STAT5 or inhibit its turnover (such as the benzotriazoles identified here) should be further evaluated as potential reactivators of latent virus.

It has been previously proposed that a single agent may not be efficient enough to completely reactivate latent HIV-1 (Laird et al., 2015). Many known LRAs were recently shown to have little to no activity when tested individually in cells isolated from aviremic patients; the strongest compound tested, bryostatin 1, only reactivated approximately 15% of cells with respect to cells treated with both PMA and ionomycin (Laird et al., 2015). Of relevance here, HODHBt, our most active benzotriazole derivative, reactivated a comparable number of latently infected cells (13% when compared to cells treated with αCD3/αCD28-coated beads). Because several groups have shown that combinations of LRAs are more effective (Abdel-Mohsen et al., 2016; Laird et al., 2015; Pache et al., 2015), it will be worthwhile to evaluate the ability of STAT5-targeting compounds to synergize with LRAs that may work through complementary pathways. For example, HDAC inhibitors may increase accessibility to the integrated HIV promoter and enhance STAT5 binding.

Reactivation of latent HIV-1 (“shock”) is not always concomitant with a reduction of the inducible latent reservoir (“kill”). For example, reactivation with the HDAC inhibitor SAHA failed to reduce the latent reservoir ex vivo unless cytotoxic T lymphocytes (CTLs) were present and antigen primed (Shan et al., 2012). Using a primary cell model of latency, we demonstrated that viral reactivation mediated by HODHBt reduced the inducible latent reservoir in vitro. Two possible scenarios can account for our results. First, a direct cytotoxic effect of HIV-1 may be responsible for this reduction, in keeping with the fact that our latency model lacks the presence of natural killer (NK) or CTL cells that could otherwise eliminate reactivated cells. Second, reactivation mediated by HODHBt may lead to transcriptional inactivation of a subset of integrated proviruses, preventing future reactivation and detection in our study. Further investigation in this area is warranted.

In our study, benzotriazole activity is dependent on prior stimulation with IL-2 in vitro. IL-2 belongs to the family of γc-cytokines, also including IL-4, IL-7, IL-9, IL-15, and IL-21, and all of which can activate STAT5 (Rochman et al., 2009). We do not know whether the cytokines present in the microenvironment in which latently infected cells reside in vivo may be sufficient to “prime” the cells to the effects of benzotriazoles, and, if not, co-administration of a γc-cytokine might be necessary. Two independent IL-2 clinical trials have shown that IL-2 administration in HIV-1 patients has little to no effect on the latent reservoir (Chun et al., 1999; Stellbrink et al., 2002). We have shown that IL-2 alone cannot reactivate latent HIV-1 in cells isolated from aviremic patients or in the cultured T_{CM} model. This is consistent with the fast turnover of STAT5 activity observed in resting CD4 T cells. Benzotriazoles have the ability to block this turnover and increase STAT5 activity, which suggests that co-administration with IL-2 may have the desirable effect of reducing the latent

reservoir. Regarding the role of other γc-cytokines, administration of IL-7 in a humanized mouse model has been shown to reactivate latent HIV-1 (Scripture-Adams et al., 2002). Furthermore, it has recently been reported that administration of IL-21 in SIV-infected macaques decreases the reservoir of latent cells harboring replication competent virus during ART (Micci et al., 2015). Finally, IL-15 or the IL-15 super agonist ALT-803 have been shown to reactivate latent HIV-1 and also enhance CTL clearance of latently infected cells (Jones et al., 2016). It will be of interest to address whether HODHBt or other benzotriazoles may also have an effect in the context of other γc-cytokines.

Finally, we observed that HODHBt induced IL-6 in PBMCs isolated from healthy donors. IL-6 is induced by STAT3 and can increase its own production in an autocrine manner (Kawano et al., 1988). One logical explanation for the presence of IL-6 is that HODHBt may also increase STAT3 activity, as suggested in our PASTAA analysis (Table S2). This result implies that STAT family proteins could share a benzotriazole-sensitive regulatory step. STATs are involved in multiple cellular processes including cell proliferation and survival, antimicrobial defense, IFN signaling, T_H1 and T_H2 differentiation, NK activity, etc. (for a review, see Miklossy et al., 2013). It will be compelling to further characterize these compounds in other cellular functions to ascertain how regulation of SUMOylation can affect other biological processes mediated by STAT activation.

In summary, benzotriazoles reactivate latent HIV-1 in a primary cell model of latency in vitro and in cells from aviremic patients ex vivo although not as effectively as αCD3/αCD28-coated beads. In light of the exciting properties of benzotriazoles, further medicinal chemistry efforts should produce new derivatives improving their potency.

EXPERIMENTAL PROCEDURES

Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: Nelfinavir, Human rIL-2 from Dr. Maurice Gately, Hoffman-La Roche (Lahm and Stein, 1985) and HIV-1_{NL4-3} from Dr. Malcolm Martin (Adachi et al., 1986). HBt and HOBt were from Sigma-Aldrich. HOAt was from ChemPep, and HODHBt was from AK Scientific. Raltegravir was from Selleckchem.

Generation of Latently Infected Cultured T_{CM} Cells

Peripheral blood mononuclear cells were obtained from Leukopaks from un-identified, healthy donors. Cultured T_{CM} and latently infected cultured T_{CM} were generated as previously described (Bosque and Planelles, 2009, 2011; Martins et al., 2016).

J-Lat Clones

J-Lat clones were provided by Jonathan Karn (Case Western Reserve University) for the 2D10 cells; Eric Verdin (University of California San Francisco) for the J-Lat 10.6 and 6.3 and Warner Greene (Gladstone Institute) for the 5A8 cells.

RNA-Seq

Poly(A)-enriched RNA-seq libraries were prepared from quiescent central-memory-like T cells incubated for 24 hr with IL-2 alone (control), with IL-2 and 100 μM HODHBt, or with αCD3/αCD28-coated beads. Paired-end reads were trimmed to remove adapters and aligned to hg19 using TopHat 2 (Kim et al., 2013), and differential expression analysis was performed using DESeq2 (Love et al., 2014). See Supplemental Experimental Procedures for additional details.

ChIP-Seq

Latently infected cultured TCM were left untreated or incubated for 3 hr with IL-2 ± 100 μM HODHBt. After 15 min, 1% paraformaldehyde crosslinking in media and sonication, ChIP was performed against STAT5A (Santa Cruz Biotechnology, sc-1081). Paired-end reads were aligned to hg19 using Bowtie 2 (Langmead and Salzberg, 2012) and compiled using MACS2 (Zhang et al., 2008). Reads which failed to map to hg19 were mapped to HIV-1 NL4-3 using Bowtie 1 (Langmead et al., 2009). See Supplemental Experimental Procedures for additional details.

QVOA Assay

Quantitative viral outgrowth assay (Q-VOA) were performed as described previously (Archin et al., 2008).

REVEAL Assay

Rapid ex vivo Evaluation of Anti-Latency activity (REVEAL) assays were performed as previously described with some variations (Spivak et al., 2015). Briefly, peripheral blood mononuclear cells were isolated from whole blood immediately after phlebotomy via density gradient centrifugation, followed by negative selection of resting $CD4^+$ (rCD4) T cells using magnetic bead separation (Miltenyi Biotec and STEMCELL Technologies). Aliquots of 5×10^6/mL resting $CD4^+$ T cells were cultured under multiple conditions for 3 days: a negative control consisting of culture medium, 30 IU/mL of IL-2, 100 μM of HODHBt, a combination of both IL-2 and HODHBt or αCD3αCD28-coated beads. 0.5% final volume of Triton was added to 350 μL of the supernatants, and virus was inactivated for 1 hr at 37°C. Unidentified samples were send to Quanterix for analysis of p24 using the Simoa™ assay (Cabrera et al., 2015; Chang et al., 2013). Samples were analyzed in duplicate.

Participant Involvement

Cells from Uninfected Blood Donors—Blood donors were 18 years and older serve as blood donors. Written informed consent was obtained from all donors. These studies are covered under the institutional review board (IRB) #67637 protocol approved by the University of Utah Institutional Review Board or blood was obtained from the Gulf Coast Regional Blood Center (Houston, TX).

Cells from Infected HIV-1^+ Donors: REVEAL Assay—Aviremic HIV-1-infected patients on ART were recruited for phlebotomy according to an approved IRB protocol

#58246 at the University of Utah. Inclusion criteria for this study required viral suppression (<50 HIV-1 RNA copies/mL) for a minimum of 6 months, ART initiation during chronic HIV-1 infection (<6 months since seroconversion), and compliance with a stable ART regimen for a minimum of 12 months per participant and provider reports. Informed consents were obtained and phlebotomies were performed in the Center for Clinical and Translational Science Clinical Services Core at the University of Utah Medical Center.

Statistics

The two-tailed paired-samples t test analysis was used to calculate p values. When appropriate, column statistics and p values were calculated. Linear regressions and p values were calculated using Prism 5 for Mac OS X software (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Benzotriazoles reactivate and decrease latent HIV-1 both in vitro and ex vivo
- Reactivation is independent of T cell proliferation or global immune activation
- Inhibition of STAT5 SUMOylation is the main target of benzotriazoles
- Benzotriazoles increase binding of STAT5 to the HIV-1 LTR

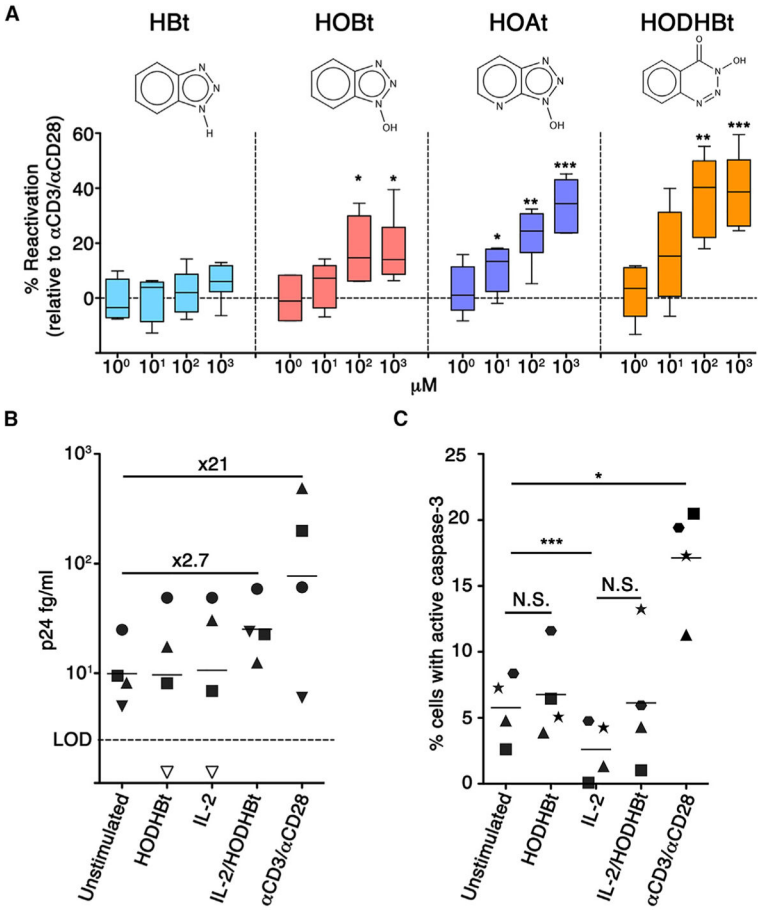

Figure 1. Novel Molecules that Reactivate Latent HIV-1 in Primary Cells In Vitro and Ex Vivo

(A) Chemical structure of compounds tested and dose response reactivation relative to αCD3/αCD28-coated beads using the cultured T_{CM} model from six different donors. Cells treated with IL-2 alone are used as a baseline, and one-sample t test analysis was used to calculate p values. Error bars indicate maximum and minimum.

(B) Viral reactivation was measured in cells isolated from aviremic patients using the REVEAL assay and a digital ELISA. Geometric means are indicated with a horizontal line, and data points below the detection limit are shown as empty symbols.

(C) Toxicity measure as caspase-3 activation in cells isolated from four aviremic patients. Means are indicated with horizontal lines. Two-sample paired t test analysis was used to calculate p values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

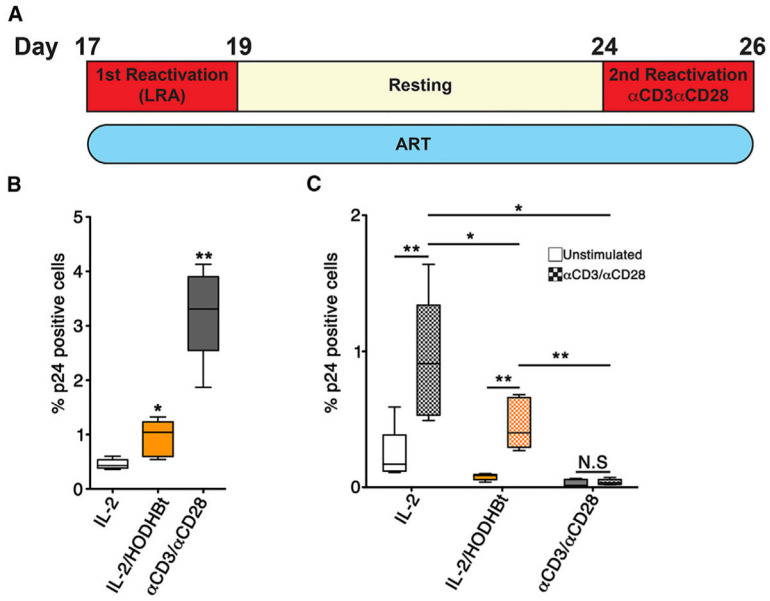

Figure 2. HODHBt Reduces the Inducible Latent Reservoir In Vitro

(A) Depletion assay timeline.

(B) Reactivation of latent HIV-1 in T_{CM} from five different donors at day 17 with IL-2 alone, IL-2 with HODHBt, or αCD3/αCD28.

(C) Subsequent HIV-1 reactivation in cells from (B) treated from days 24–26 with αCD3/αCD28 (*$p < 0.05$; **$p < 0.01$). Error bars indicate maximum and minimum.

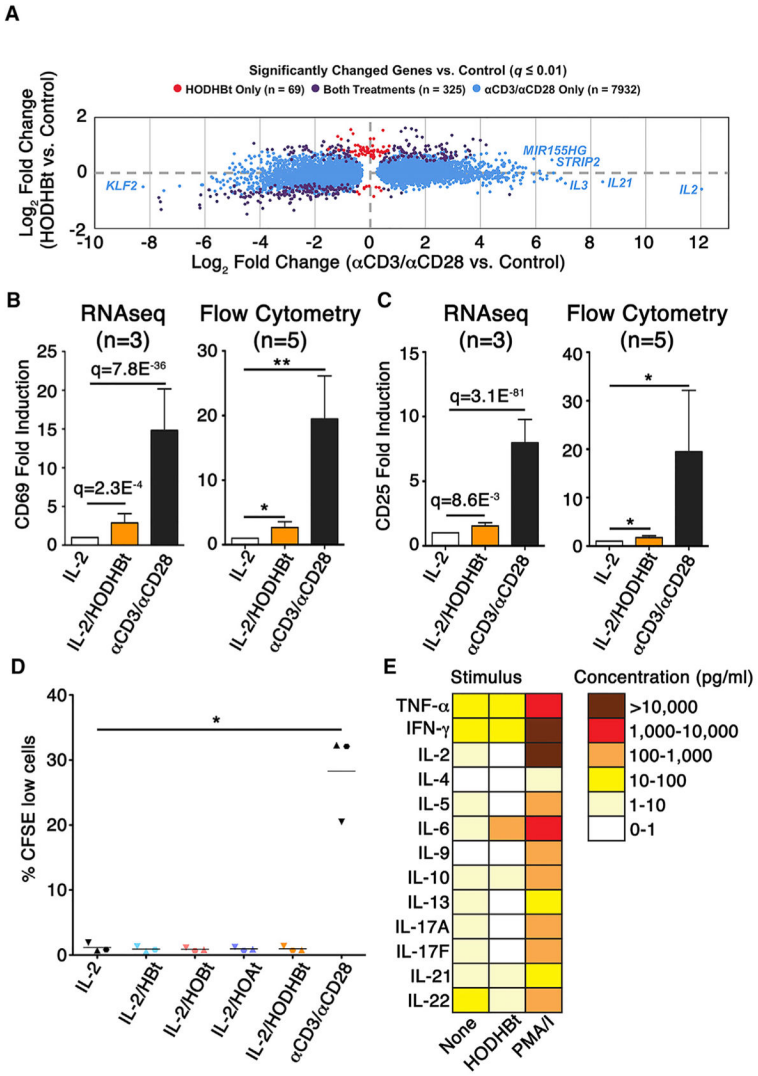

Figure 3. Effects of HODHBt on Gene Expression, Surface Markers, Proliferation, and Cytokine Release

(A) Differential expression analysis of poly(A)-selected RNA-seq from three donors in matched primary cultured T_{CM}. False-discovery-rate-adjusted p values (q values) were used to identify genes whose expression was significantly altered ($q \leq 0.01$). Log_2 fold changes (treatment versus control) of genes whose expression was significantly altered by HODHBt only (red), αCD3/αCD28 only (blue), or both treatments (purple) were compared.

(B and C) Fold induction of CD69 expression (B) or CD25 expression (C) measured by RNA-seq (left) or flow cytometry (right). Fold induction relative to cells treated with IL-2 was calculated using the mean fluorescence intensity (MFI). Error bars indicate SD. Significant q values and p values over cells treated only with IL-2 are indicated.

(D) Cellular proliferation of cultured T_{CM} from three donors measured by dilution of CFSE after 48 hr of treatment.

(E) PBMCs were treated as indicated and assayed for cytokine concentrations. Data are the average effect from five healthy donors (*$p < 0.05$; **$p < 0.01$).

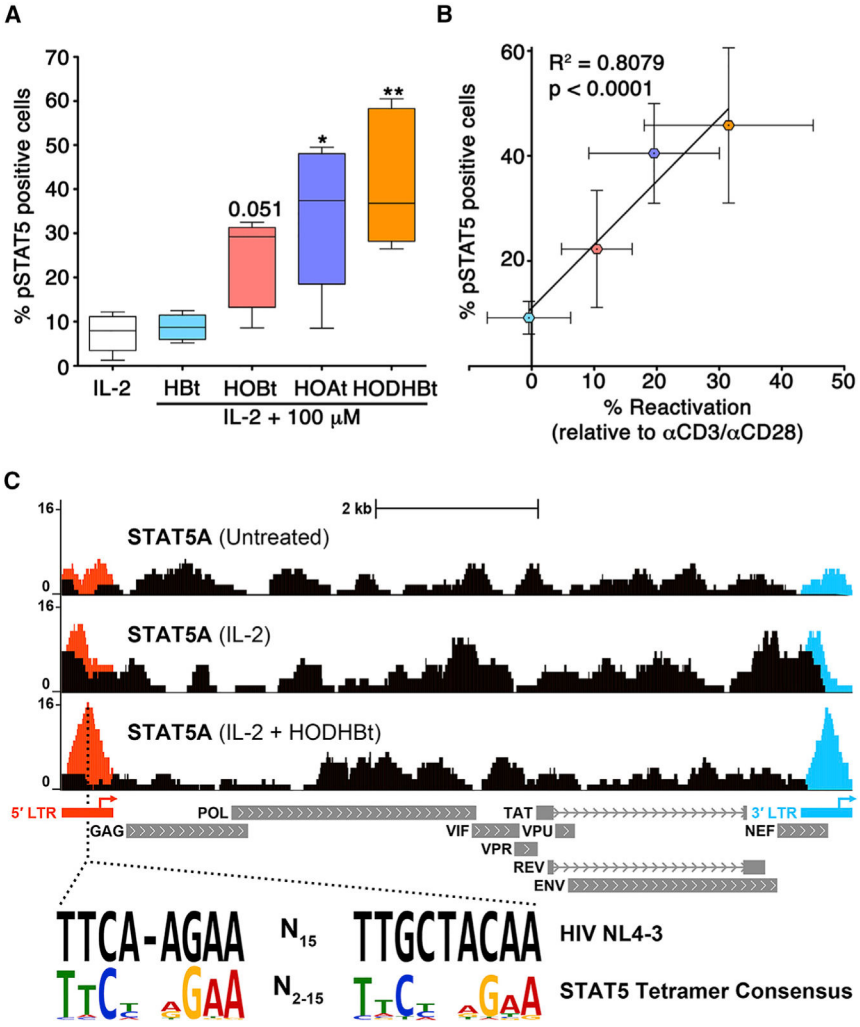

Figure 4. HODHBt Sustains pSTAT5 and Binding to the HIV-1 LTR

(A) Level of pSTAT5 positive cells 24 hr after treatment of cultured T_{CM} in five different donors. Error bars indicate maximum and minimum. Two-tailed paired-samples t test analysis was used to calculate p values (*$p < 0.05$; **$p < 0.01$).

(B) Levels of pSTAT5 positive cells at 24 hr were correlated with levels of HIV-1 reactivation at 48 hr. Samples were grouped by treatment. Error bars indicate SD. Correlation and p value were calculated using all 20 data points.

(C) STAT5A ChIP-seq tracks over the HIV-1 genome of latently infected cultured T_{CM} cells left untreated, or treated with IL-2 or IL-2 plus HODHBt for 3 hr. All tracks were normalized to the average number of hg19-mapped sequence reads. Reads which could not be definitely assigned to either LTR are shown in both locations (5′, red; 3′, blue). The STAT5-occupied region located in the HIV-1 LTR (318–350) was compared with the consensus tetramer sequence (Lin et al., 2012).

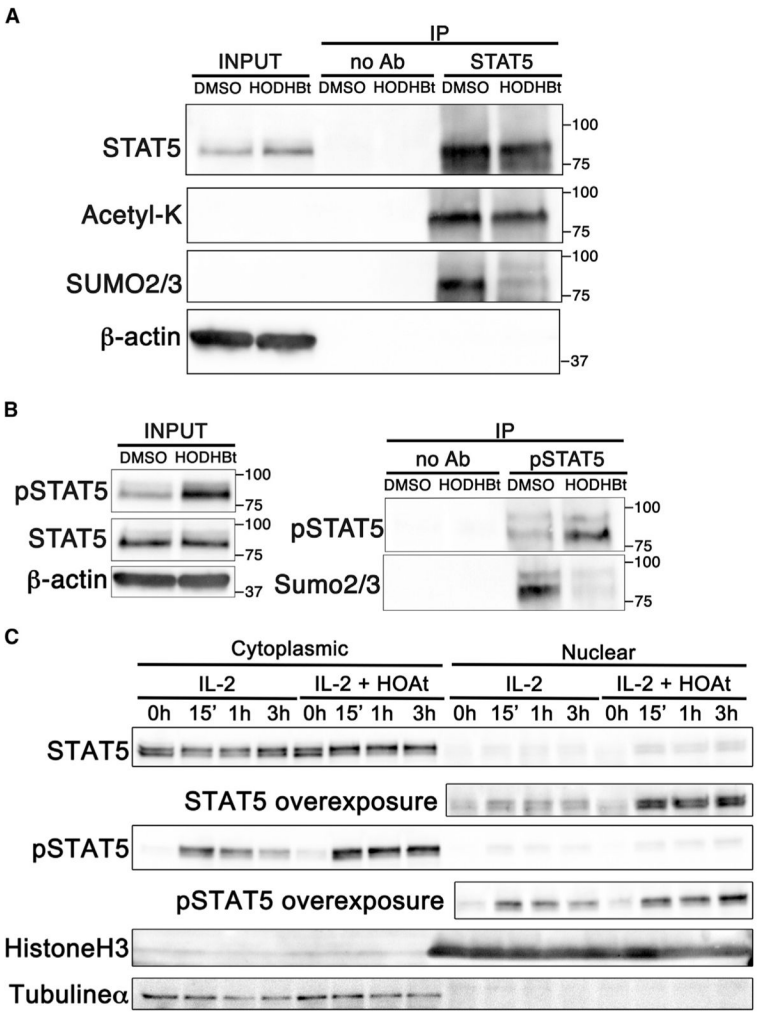

Figure 5. HODHBt Inhibits SUMOylation of STAT5

(A) STAT5 was immunoprecipitated and analyzed by immunoblot for acetylated lysine (Acetyl-K) and SUMO2/3 in cultured T_{CM} after treatment with IL-2 for 24 hr in the presence or absence of 100 μM HODHBt.

(B) Levels of STAT5 and pSTAT5 in cultured T_{CM} after treatment with IL-2 for 24 hr in the presence or absence of 100 μM HODHBt as analyzed by immunoblot. pSTAT5 was immunoprecipitated and analyzed by immunoblot for SUMO2/3.

(C) Levels of STAT5 and pSTAT5 were analyzed in cytoplasmic and nuclear fractions of cultured T_{CM} treated with IL-2 in the presence or absence of 100 μM HOAt. Data are representative of two donors.

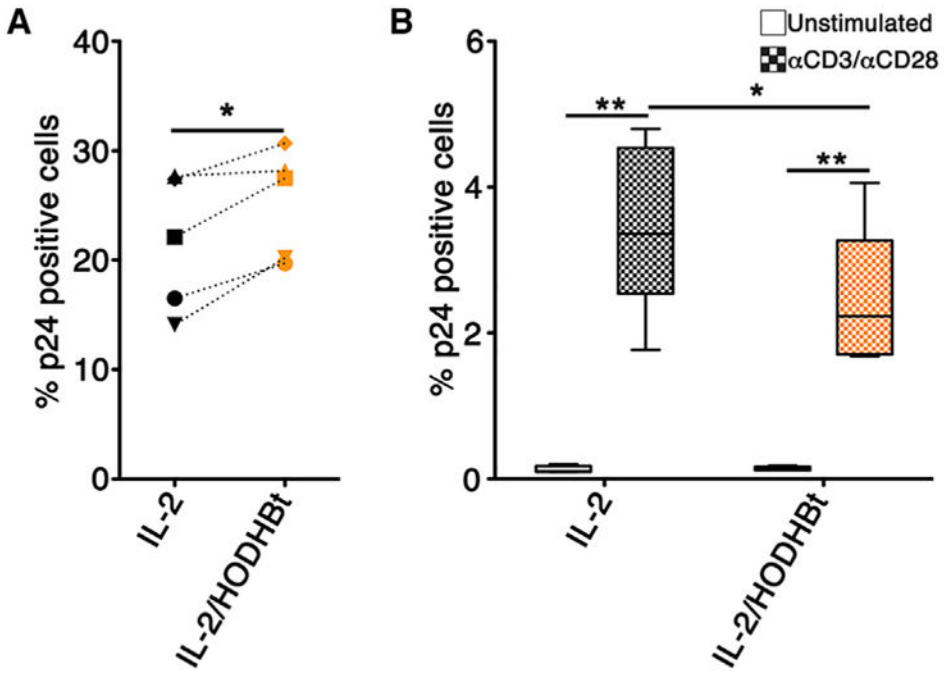

Figure 6. HODHBt Blocks the Establishment of Latency

(A) At day 10, infected cells were treated with IL-2 or IL-2 plus HODHBt for 3 days and levels of p24 positive cells were assess by flow cytometry in five different donors. Two-tailed paired-samples t test analysis was used to calculate p values. (*$p < 0.05$).

(B) Latently infected cells were treated with IL-2 or αCD3/αCD28 and levels of p24 positive cells were assessed by flow cytometry in the five different donors from (A). Error bars indicate maximum and minimum. Two-sample paired t test analysis was used to calculate p values (*$p < 0.05$; **$p < 0.01$).

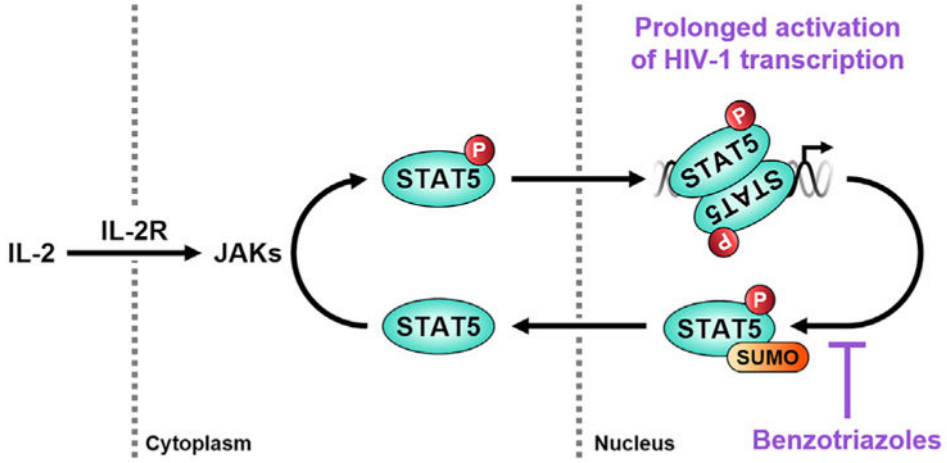

Figure 7. Benzotriazoles Reactivate HIV-1 through STAT5

In cells treated with IL-2, pSTAT5 is inactivated by a mechanism involving SUMOylation. HODHBt blocks SUMOylation of STAT5, increasing its transcriptional activity.